Bleeding tendency and health-related quality of life in carriers of haemophilia

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Äntligen…!

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ABSTRACT

Haemophilia A and B are X-linked disorders caused by impaired synthesis of coagulation factors VIII and IX, respectively. Women who carry the haemophilia trait have about 50 % of normal factor levels. Due to skewed X-chromosome inactivation, factors in carriers may however range from levels corresponding to those in men with haemophilia up to normal levels. One hundred and twenty six haemophilia carriers and 90 controls were included in the study. In **paper I**, bleeding tendency was evaluated with a standardised bleeding assessment tool. We found increased bleeding tendency among the carriers of haemophilia A and B, compared to the control group. The bleeding tendency was weakly correlated to FVIII levels. In **paper II**, health-related quality of life (HRQOL) in haemophilia carriers was compared to a control group and the normative population. HRQOL was evaluated with the Short-Form 36 questionnaire. Symptomatic carriers had lower scores in the General Health, Social Functioning and Mental Health domains, compared to the control group. These differences disappeared when comparisons were made with the normative population. **Paper III** demonstrates that thrombin generation potential, evaluated by the calibrated automated thrombography method (CAT), did not differ significantly in symptomatic and asymptomatic carriers of haemophilia A. The results of **paper IV** indicate that there was no association between bleeding tendency in haemophilia A carriers and genotype, evaluated by comparison of null and non-null mutations. In conclusion, the results suggest that carriers of haemophilia may have increased bleeding tendency, especially during haemostatic stress. Carriership did not affect HRQOL in comparison to the normative population. Factor levels as well as thrombin generation capacity appear to be inadequate for prediction of bleeding tendency in carriers. The bleeding tendency in haemophilia A carriers was not influenced by the genotype.

Keywords: Carriers of haemophilia, bleeding, SF-36, thrombin generation, genotype.
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SAMMANFATTNING PÅ SVENSKA

Bakgrund. Hemofili A och B ärvs X-kromosombundet och orsakas av medfödd brist på eller avsaknad av koagulationsfaktor VIII respektive IX. Kvinnor som bär på anlaget för hemofili har vanligen ca 50 % av normal faktornivå. På grund av skev X-kromosom inaktivering kan faktornivån hos bärare variera från låga, i motsvarande nivå som hos män med mild hemofili, upp till normal värden.

Frågeställning. I avhandlingen undersöktes om bärare har en ökad blödningsbenägenhet respektive påverkad hälsorelaterad livskvalitet jämfört med en kontrollgrupp respektive en svensk normativ population. Vidare undersöktes om blödningsbenägenheten var korrelerad till koagulationsfaktornivå. Därtill studerades om blödningsbenägenheten hos bärare av hemofili A kunde värderas med hjälp av trombingenereringsförmåga respektive utifrån genotyp.

Metodik. Etthundratjugosex bärare och nittio kontrollpersoner deltog i studien. Blödningsbenägenheten värderades med hjälp av ett standardiserat intervjuverktyg (BAT) och hälsorelaterad livskvalitet (HRQOL) studerades med Short Form-36 (SF-36). Förmågan att generera trombin mättes med CAT-metod. Sjukdomsorsakande mutationer i genen för FVIII delades in i null respektive non-null mutationer.

Resultat. Vi fann en ökad blödningsbenägenhet hos bärare av hemofili A och B jämfört med kontrollgruppen. Blödningsbenägenheten hos bärare av hemofili A korrelerade svagt till faktor VIII nivå. Bärare med ökad blödningsbenägenhet hade lägre SF-36 poäng inom domänerna för Allmän hälsa, Social funktion och Psykiskt välbefinnande, jämfört med kontrollgruppen. Dessa skillnader försvann vid jämförelse med den svenska normativa populationen. Blödningsbenägenhet hos bärare av hemofili A kunde inte värderas utifrån trombingenereringsförmåga och var inte associerad med genotyp uppdelad i null och non-null mutationer.

Slutsatser. Bärare av anlag för hemofili A och B kan ha en ökad blödningsbenägenhet, framför allt i situationer som ställer ökat krav på blodstillningsförmågan såsom vid operationer och trauma. Faktornivåer förefaller inte kunna förutspå blödningsbenägenhet på ett tillräckligt adekvat sätt. Bärarskap påverkade inte HRQOL i jämförelse med den normativa svenska populationen. Trombingenereringsförmåga skilde inte signifikant mellan bärare av hemofili A med och utan ökad blödningsbenägenhet. Genotyp hos bärare av hemofili A var inte associerad till blödningsbenägenhet.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I Olsson A, Hellgren M, Berntorp E, Ljung R, Baghaei F.
Clotting factor level is not a good predictor of bleeding in carriers of haemophilia A and B
Blood Coagulation and Fibrinolysis 2014; 25: 471-475

II Olsson A, Hellgren M, Berntorp E, Baghaei F.
Association between bleeding tendency and health-related quality of life in carriers of moderate and severe haemophilia
Haemophilia 2015; 21: 742-746

III Olsson A, Hellgren M, Berntorp E, Holmström M, Baghaei F.
Bleeding phenotype in carriers of haemophilia A does not correlate with thrombin generation
Haemophilia 2015; 21: e111-e113

IV Olsson A, Ljung R, Hellgren M, Berntorp E, Baghaei F.
Phenotype and genotype comparisons in carriers of haemophilia A
Haemophilia 2016; 1-3

CONTENT

ABBREVIATIONS

APTT	Activated partial thromboplastin time
AT	Antithrombin
BAT	Bleeding assessment tool
BMI	Body mass index
BQ	Bleeding questionnaire
BP	Bodily Pain
BS	Bleeding score
CAT	Calibrated automated thrombin generation assay
CNS	Central nervous system
CTI	Corn trypsin inhibitor
DDAVP	Desmopressin
ETP	Endogenous thrombin potential
F	Factor
F8	Factor VIII gene
F9	Factor IX gene
FVIII	Coagulation factor VIII
FVIII:C	Factor VIII coagulant activity
FIX	Coagulation factor IX
FIX:C	Factor IX coagulant activity
GH	General Health
GPIb	Glycoprotein Ib
GPIIbIIIa	Glycoprotein IIbIIIa
GPVI	Glycoprotein VI
HA	Haemophilia A
HB	Haemophilia B
Hb	Haemoglobin
HRQOL	Health-related quality of life
HTC	Haemophilia treatment centre
ISTH	International Society of Thrombosis and Haemostasis
IU	International unit
MCS	Mental Component Summary
MH	Mental Health
NA	Not applicable
ns	Non-significant
PAI-1	Plasmin activator inhibitor 1
PCR	Polymerase chain reaction
PCS	Physical Component Summary
PF	Physical Functioning

PPH	Post-partum haemorrhage
PPP	Platelet-poor plasma
PT (INR)	Prothrombin time (international normalized ratio)
RE	Role-Emotional
RP	Role-Physical
SD	Standard deviation
SF	Social Functioning
SF-36	Short Form 36
TF	Tissue factor (thromboplastin)
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
TGA	Thrombin generation assay
t-PA	Tissue plasminogen activator
VT	Vitality
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:RCo	von Willebrand Ristocetin cofactor activity
WHO	World Health Organisation
XCI	X-chromosome inactivation

1 INTRODUCTION

1.1 Haemostasis

Haemostasis is a protective mechanism, responsible for keeping blood in a fluid state in the vessels as well as preventing blood loss at sites of injury. It is essential that haemostasis is activated at the right time, to the right extent and at the exact location. Haemostasis is divided into primary haemostasis, including vascular constriction and the forming of a platelet plug; secondary haemostasis or coagulation; and fibrinolysis, in which the formed clot is dissolved [1].

1.1.1 Primary haemostasis

When the endothelial layer is disrupted due to injury, local vasoconstriction slows the blood flow and platelets can adhere to the vessel wall. Platelet receptors glycoprotein 1b (GPIb) and glycoprotein VI (GPVI) bind with von Willebrand factor (VWF) and collagen, respectively, to anchor the platelets to the sub-endothelium [2, 3]. The platelets are thus activated. Following activation, the platelets change shape, active substances are released from granules and the fibrinogen receptors glycoprotein IIbIIIa (GPIIbIIIa) are expressed [4]. Additional platelets can then aggregate via inter-platelet fibrinogen bridges and a platelet plug is formed [5, 6].

1.1.2 Coagulation

The platelet and its importance in haemostasis were discovered at the end of the 19th century [7]. The first clotting model was presented after the turn of the century. The model contained four components: thromboplastin is released from the injured vascular tissue and converts prothrombin into thrombin in the presence of calcium; thrombin then converts fibrinogen into fibrin and a clot is formed [8]. This four component clotting model could not explain the complexity of coagulation and so research continued. In the mid-twentieth century many of the remaining coagulation factors (F), i.e. FV, FVII, FVIII, FIX and FXI were identified. It was recognized that factor deficiencies may lead to more or less severe bleeding disorders. In the 1960s a model was constructed representing coagulation as a waterfall. In this model, known as the coagulation cascade, each proenzyme is converted to its active state by an upstream-activated coagulation factor. The coagulation cascade is divided into an intrinsic, contact-activated pathway and an extrinsic pathway activated through tissue factor (TF). The pathways converge into a common pathway in which activated FX (FXa) converts prothrombin into thrombin [9, 10].

The current cell-based model of haemostasis indicates that the coagulation reactions occur as overlapping steps on cell surfaces rather than as a cascade [11, 12]. Procoagulant reactions are localized on phospholipid cell surfaces, forming and maintaining the platelet and fibrin plug at the site of injury. The cell-based model of coagualtion is described in three separate phases: the initiation phase, the amplification phase and the propagation phase (fig 1).

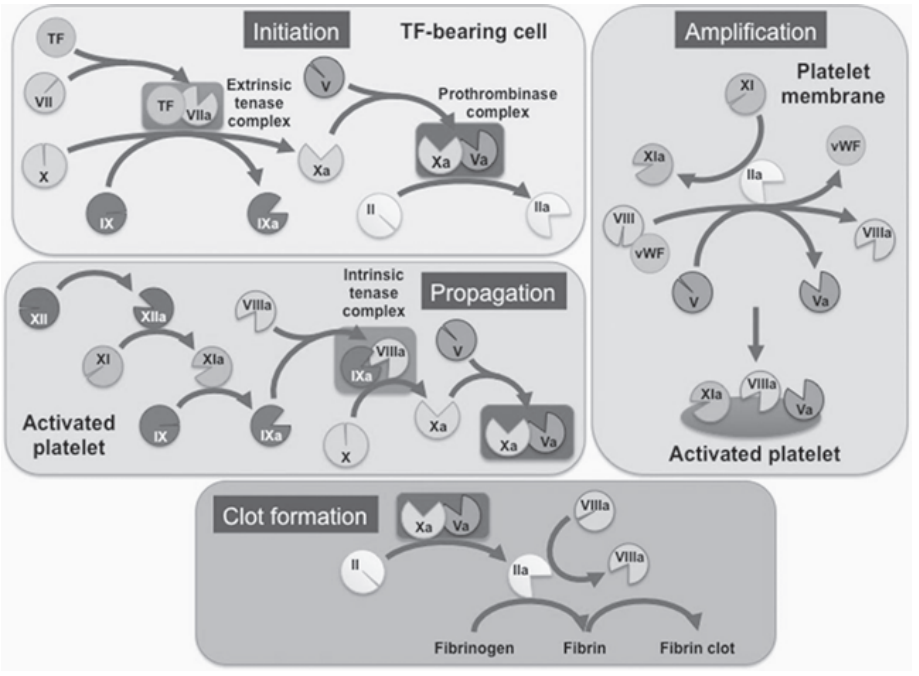

Figure 1. Cell-based coagulation. Roman numerals indicate inactive and active coagulation factors. (Reprinted with permission from Oxford University Press) [16]

The initiation phase occurs on TF-bearing cells. Activated FVII (FVIIa), bound to TF, activates small amounts of FIX and FX. FXa binds to FVa and converts a small amount of prothrombin to thrombin, initiating coagulation.

The amplification phase occurs on the surface of activated platelets. The small amount of thrombin generated on the TF-bearing cells activates platelets that have adhered to the site of injury. Furthermore, thrombin cleaves FV, FVIII and FXI into their activated forms.

The propagation phase occurs on the surface of activated platelets. The tenase complex (FIXa/FVIIIa) formed on the platelet surface rapidly starts to generate FXa. FXa binds to FVa to form the prothrombinase complex that cleaves prothrombin to thrombin. This results in a burst of thrombin generation (TG), leading to cleavage of fibrinogen into fibrin to form a clot. Thrombin activates FXIII, which enables strengthening of the fibrin clot [13].

In order to prevent uncontrolled clot formation at the site of an injury, coagulation must be restrained. Restraint in terms of space is based on coagulation only taking place on pro-coagulant cell surfaces, while time restraint is generated by inhibitor pathways. The three major inhibitors are tissue factor pathway inhibitor (TFPI), that inhibits TF-FVIIa as soon coagulation has been initiated; antithrombin, that inhibits thrombin, FIXa and FXa; and activated protein C, that inactivates FVa and FVIIIa in the presence of protein S [14].

1.1.3 Fibrinolysis

Fibrinolysis is initiated once the fibrin clot starts to form. The clot is lysed by plasmin originating from fibrin-bound plasminogen. The enzyme tissue plasminogen activator (t-PA) is the main activator of plasminogen. t-PA-induced generation of plasmin is controlled by specific inhibitors, the most important of which is plasminogen activator inhibitor 1 (PAI-1) [15].

1.2 Haemophilia

Haemophilia is one of the oldest recognized hereditary diseases. Throughout the centuries, there have been reports of boys and men who have bled to death from minor wounds and procedures [17]. The first modern description of haemophilia was by J.C. Otto, an American physician, who in 1803 described a family consisting of seemingly healthy women and men suffering from a congenital bleeding disorder [18].

Haemophilia A and B are rare bleeding disorders caused by lack or deficiency of functioning FVIII or FIX. Approximately 1 000 Swedish men and boys have haemophilia [19]. The ratio between haemophilia A and B is 4:1. The characteristic bleeding manifestation in haemophilia is repeated bleeding in joints, leading to arthropathy. Haemophilia is classified as severe, moderate or mild, according to coagulation factor activity in the blood. In severe haemophilia the factor level is < 0.01 kIU/L and joint and muscle bleeding, as well as internal bleeding, may occur spontaneously or after minor trauma. In moderate haemophilia the factor level is 0.01 - 0.05 kIU/L but bleeding may occur spontaneously in this condition as well. In mild haemophilia the factor level is > 0.05 to 0.40 kIU/L and spontaneous bleeding is rare, but may occur after minor trauma or surgery [20, 21]. By definition, normal plasma contains 1 IU/mL of each factor. Normal ranges should be established locally, but the lower limit is often approximately 0.50-0.60 kIU/L. Replacement therapy in haemophilia has improved substantially during the last 40-50 years. Nowadays, patients with severe and moderate haemophilia living in high-resource nations are treated with prophylactic intravenous recombinant FVIII or FIX concentrate two to four times a week to prevent bleeding. Patients with mild haemophilia are usually not given prophylactic treatment but are instead treated with factor concentrate as needed [22]. In patients with mild haemophilia A, desmopressin (DDAVP), a synthetic anti-diuretic hormone analogue, is a treatment option since it stimulates the endogenous release of FVIII and VWF [23]. Prophylactic treatment with factor concentrate has dramatically reduced morbidity and mortality. In the 1950s and 1960s, the median life expectancy among men with severe haemophilia was 26 years in Sweden. Today, life expectancy is almost the same as for the general population [24, 25].

1.3 Inheritance and genetic aspects of haemophilia

Haemophilia A and B are X-linked recessive disorders. Women who inherit the gene are carriers of the disease trait, while men who inherit the gene have haemophilia. All daughters of a haemophilic father will become obligatory carriers. There is a 50 % chance that a carrier mother will transmit the defective gene to a son, who inherits haemophilia, or to a daughter, who inherits the trait (Fig 2).

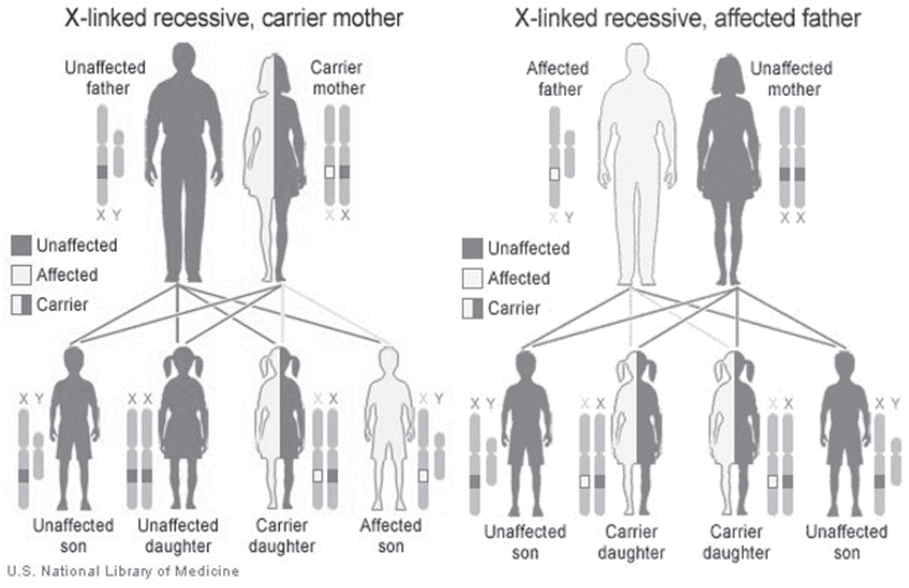

Figure 2. Inheritance patterns of haemophilia (reprinted with permission).

One third of new cases of haemophilia A and one fifth of new cases of haemophilia B are sporadic rather than familial and are due to a de novo mutation [26]. In about 80 % of the sporadic cases, the mutation is also present in the carrier mother; in the majority of these cases the mutation has occurred in the germ cells of a phenotypically normal grandfather [27].

The phenomenon of somatic mosaicism in carriers (onset of mutation in early embryogenesis) is fairly common and has clinical implications. A carrier may appear to be a non-carrier after testing, although she still risks giving birth to a haemophilic boy [28]. Genetic counselling, carrier testing and prenatal diagnosis of haemophilia are integrated parts of comprehensive care for this condition. In addition to assessment of the coagulation factor level, molecular genetic analysis is required to determine carrier status. A normal FVIII:C or FIX:C level does not exclude carriership. Direct identification of the mutation known to cause haemophilia in the family is preferred, but linkage analysis may be performed instead [29, 30].

The genes of FVIII (F8) and FIX (F9) are located on the distal part of the long arm of the X chromosome at Xq28 and Xq27, respectively. The F8 gene, one of the larger human genes, was cloned in 1984 [31, 32]. The F9 gene was cloned in 1982 [33, 34]. After the respective genomic structures were determined, several mutations were identified, but it was only when polymerase chain reaction (PCR) testing became routine in the late 1980s that full mutation screening became feasible. The online FVIII Variant Database and FIX Variant Database currently report over 2 000 unique mutations for haemophilia A and over 1 000 unique mutations for haemophilia B [35, 36]. Since the 1990s, several countries, including Sweden, have developed national mutation databases containing pedigrees and mutation details for haemophilia families. This resource is invaluable for carrier and prenatal screening.

The mutations in haemophilia may be divided into point mutations, insertions, deletions and rearrangements/inversions. In haemophilia A the single most important defect is the intrachromosomal inversion involving intron 22 of the F8 gene, which results in about 45 % of all severe haemophilia A cases worldwide [37]. A similar inversion, involving intron 1 of the F8 gene, is reported in about 1-2% of patients with severe haemophilia A [38]. There are no rearrangements in haemophilia B equivalent to the large inversions seen in haemophilia A. Instead, essentially every haemophilia B family has its own unique mutation. Point mutations are the most common gene defect and comprise missense mutations (a different amino acid is encoded), nonsense mutations (a mutation leading to a stop codon and abrupt termination of translation) and splice site point mutations (that corrupt or create a new mRNA splice site). Deletions and insertions generally lead to severe and occasionally moderate disease. Nonsense mutations lead to severe disease, but the disease severity of missense and sometimes splice site mutations depends on the location and the particular function of the amino acid affected [39].

X-chromosome inactivation (XCI) is the process by which one of the two X-chromosomes present in females is inactivated to achieve equivalency for X-linked genes between XX females and XY males. XCI occurs in early embryonic life and is then stably inherited through the subsequent somatic cell divisions [40]. XCI is defined as skewed if the same allele is inactivated in 75-80 % of the cells [41]. Genetic and epigenetic factors have been determined to be involved in establishing and maintaining XCI, but the mechanisms are not yet completely understood [42, 43].

1.4 Carriers of haemophilia

Due to XCI, haemophilia carriers are expected to have factor levels corresponding to about 50 % (0.50 kIU/L) of those found in normal individuals [44, 45]. This level is generally considered to be sufficient for normal haemostasis [46]. However, a wide range of clotting factor levels is observed in carriers. Factor levels may range from very low (< 0.10 kIU/L), the level found in males with mild haemophilia, to the upper normal limit (< 2.0 kIU/L) [47].

Bleeding tendency in carriers has been previously studied, but due to the lack of standardised instruments, different in- house questionnaires have been constructed for that purpose. Mauser Bunschoten et al. conducted a study in the Netherlands in 1988, concluding that bleeding tendency was correlated to reduced factor levels [48]. Miesbach et al. reported increased bleeding tendency in 19 carriers of haemophilia A with a mean FVIII:C of > 0.50 kIU/L [49]. There have also been case reports on increased bleeding tendency among carriers with extremely low factor levels [47, 50, 51]. In 2006 Plug et al. published the results of a large study in which 274 carriers filled out a questionnaire concerning bleeding symptoms. Data on previous FVIII:C and FIX:C levels were available in most of the cases. The results suggest an increased bleeding tendency among carriers, especially after medical interventions or trauma, including at factor levels within the lower normal range [52]. Since we recognise that increased bleeding tendency in carriers is a clinical problem, we wanted to investigate the Swedish carrier population using a standardised bleeding assessment tool (BAT).

2 AIMS OF THE STUDIES

The overall aim was to conduct clinical and basic research to increase our understanding of bleeding tendency in carriers of haemophilia and thereby improve the care of these women. The specific aims were as follows:

To investigate bleeding tendency among carriers of severe and moderate haemophilia A and B, compared to a control group, and to correlate bleeding symptoms with levels of FVIII:C and FIX:C (Paper I)

To investigate the health-related quality of life in carriers of haemophilia A and B, compared to the normative Swedish female population (Paper II)

To investigate whether thrombin generation capacity reflects bleeding tendency in carriers of severe and moderate haemophilia A (Paper III)

To determine possible associations between genotype, i.e. null vs non-null mutations, and bleeding tendency in carriers of severe and moderate haemophilia A (paper IV)

3 METHODS

Information on study populations, collection of data and laboratory and statistical methods are reported in Papers I-IV. In this chapter, selected methods are described in detail.

3.1 Study populations

The studies were approved by the Regional Research Ethics Committee at Gothenburg University (Registry number 450-09). Informed consent was obtained from all participants. The study populations were intended to include the known population of carriers of severe and moderate haemophilia A and B in Sweden. Relative to the number of men with severe and moderate haemophilia, there ought to be approximately 450 carriers in Sweden [53]. Two hundred and eighty-one adult potential carriers were identified via the haemophilia treatment centres (HTC) at Sahlgrenska University Hospital, Karolinska University Hospital and Skåne University Hospital/Malmö.

Paper I

During 2011, 281 adult potential carriers were invited by mail to participate in the study. One hundred and forty-five women agreed to participate. One hundred and thirty-six women declined or did not answer the invitation. Eleven women were excluded since DNA mutation analysis failed to confirm carriership, and eight women dropped out after providing informed consent. Each participating carrier was asked to suggest a female friend with neither a bleeding disorder nor a family history of bleeding disorders to be part of the control group. Ninety controls were included.

Paper II

Two of the carriers of severe haemophilia A failed to complete the Short Form 36 questionnaire (SF-36) and were excluded from participation. One hundred and twenty-four carriers and 90 controls participated. The results were compared to the results in the general female population reported in the Swedish SF-36 Health Survey (n = 4 582) [54, 55].

Papers III and IV

Carriers of haemophilia A were included in Studies III and IV (n = 106). One carrier of severe haemophilia was excluded from Study III and one was excluded from Study IV, due to lack of plasma and inability to classify an existing splice site mutation according to the protocol, respectively.

3.2 Assessment of bleeding tendency (Papers I-V)

During the last decade, there has been interest in developing a tool to objectively record and clinically evaluate bleeding symptoms. The Vicenza Bleeding Questionnaire (BQ), published in 2005, established the framework and scoring key that were later modified into the current BAT developed by the International Society of Thrombosis and Haemostasis (ISTH-BAT) [56-58]. The original Vicenza BQ was validated to distinguish type 1 von Willebrand disease (VWD) from the normal population [56]. The BATs evolved from the Vicenza BQ are based on interviews and recollections to report bleeding tendency in a standardised manner, but are not validated for a specific diagnosis. The BAT used in this thesis was published in 2008 and is a modified and condensed version of the original Vicenza BQ [59]. The BAT evaluates the severity and frequency of twelve bleeding symptoms and adds them to create a quantitative, summative bleeding score (BS). The twelve bleeding symptoms are graded from 0 to 4, according to severity. A lack of excessive bleeding during surgery, delivery or tooth extraction results in a negative score in that specific category. Only the worst-case scenario is scored and any situation in which prophylactic treatment was given is excluded. Normal BS was determined by interviewing 100 healthy individuals and ranged from -3 to 3 [59]. **Appendix A**.

3.3 Health-related quality of life measurement (Paper II)

Generic health-related quality of life (HRQOL) instruments allow comparisons between different populations, as well as with the general population, regardless of health disorder. Disease-specific instruments are used to detect changes and are suitable for evaluation of treatment. There are validated disease-specific instruments for patients with haemophilia, but not for carriers [60, 61]. The generic instrument SF-36 was published in 1992 [62]. It has since been translated into and validated for several languages, and specific normative data for the general population in each country have been developed [63, 64]. The questionnaire is self-administered and takes about 10 minutes to complete. It consists of 36 questions representing eight domains, in addition to overall physical and mental health component summary scores (PCS and MCS) [65]. The results in each domain are coded and transformed into scale scores from 0 (worse possible health) to 100 (best possible health) [55, 65]. The scale scores may be transformed into norm-based scores, which reflect the number of standard deviations (SD) with which a studied population differs from the age- and gender-specific mean in the normative population. Norm-based scores permit comparison across scales and populations. **Appendix B**.

3.4 FVIII and FIX coagulant activity measurements (Papers I, III, IV)

FVIII:C was analysed with two methods, the one-stage clotting assay and the two-stage chromogenic assay. The one-stage assay is based on the ability of a sample to normalise or shorten the delayed clotting of FVIII-deficient plasma in an activated partial thromboplastin time (APTT)-based test system. Reference plasma calibrated in international units is used to verify the FVIII:C level [66, 67]. In the chromogenic assay, patient plasma is mixed with thrombin, FX and FIXa reagents. FVIII that is activated to FVIIIa converts FX to FXa. FXa cleaves a chromogenic substrate and the absorbance is directly proportional to the amount of FVIII:C [66, 67].

The chromogenic assay may be considered to be the more accurate of the two since it does not depend on deficient plasma and the APTT assay. A discrepancy between the methods is seen in about 20 – 30 % of the patients with non-severe haemophilia A, for which certain mutations of the F8 gene are at least partly responsible [68-70]. Most common is an, up to two-fold, lower value with the chromogenic assay [71-73]. The opposite does occur, but bleeding symptoms seem to be consistent with the chromogenic results [71, 74]. In this thesis, reported results are from the chromogenic FVIII:C assay.

FIX:C was measured with a one-stage clotting assay. The principle is the same as in one-stage FVIII:C measurements, but FIX-deficient plasma is used instead, and the results are calibrated against a reference FIX plasma sample [66].

3.5 Thrombin generation (Papers III - IV)

The thrombin generation assay (TGA) is considered to reflect the global haemostatic capacity in vitro [75, 76]. Routine clotting assays, APTT and prothrombin time (PT) measure time to initiation of clot formation and do not include the thrombin burst. The amount of thrombin generated is determined by the combined activity of pro- and anti-coagulation factors, which means that the assay evaluates both hyper- and hypo-coagulable conditions [77]. The original TGA was published in the 1950s. The method required continuous sub-sampling and was technically difficult to perform [78]. Since the 1980s Hemker et al. have developed the technique, resulting in the calibrated automated thrombin generation assay (CAT) [79, 80].

The CAT is based on a slow-acting fluorogenic substrate that enables the continuous measurement of TG. The substrate is not affected by the turbidity of clot formation or the presence of platelets. The relationship between thrombin activity and the fluorescent signal is not linear, due to substrate consumption and the inner filter effect of fluorescence measurements. This problem is overcome by comparing the fluorescent signal to a constant thrombin calibrator with known thrombin activity in a non-clotting plasma sample [80]. The results are presented as a thrombogram from which specified parameters are calculated (lag-time, time to peak, peak thrombin concentration and endogenous thrombin potential (ETP)) (Fig 3). Peak thrombin concentration and ETP are considered to best reflect a reduced clotting factor level [77].

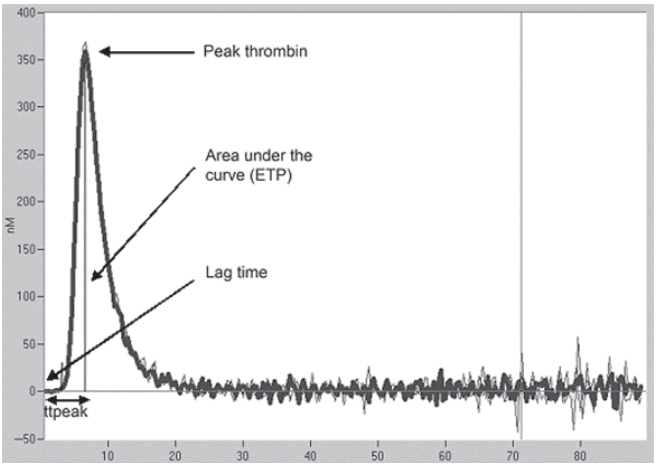

Figure 3. Parameters of the thrombogram: lagtime (min), time to peak (min), peak thrombin (nM), endogenous thrombin potential (ETP) (nM/min). (Reprinted with permission from Blackwell Publishing Ltd) [81]

3.6 Statistical methods

The Mann Whitney U test was performed to compare differences between groups (Papers I-IV). Fisher´s exact test was used to compare baseline characteristics and the prevalence of symptoms between groups (Papers I-II). The Bonferroni correction was applied to adjust for multiple comparisons. The Spearman rank correlation was used to calculate correlations between variables (Papers I and IV). In Paper II the scale scores of the SF-36 were transformed into Z-scores by subtracting the mean scale score of the normative population sample (matched for age) from the scale score of the study group and dividing the difference by the SD in the normative population sample.

Statistical analyses in Papers I, III and IV were performed with SPSS software version 19-22 (SPSS Inc, Chicago, IL, USA). Statistical analyses in Paper II were performed with SAS software version 9.0 (SAS Institute Inc, Cary, NC, USA).

4 RESULTS

4.1 Bleeding tendency in carriers of haemophilia (Paper I)

Potential carriers of severe and moderate haemophilia A and B (n = 281) were identified via clinical data from the three HTCs in Sweden. One hundred and thirty seven (49 %) chose to participate. DNA analyses were performed to confirm carriership, disclosing that 126 (92 %) women were eligible for the study. These carriers of severe and moderate haemophilia and 90 controls were included in the study. One hundred and seven of the participants were carriers of haemophilia A and 19 were carries of haemophilia B. The group of non-participants (n = 144) included women who decided not to participate and women who dropped out after providing informed consent (n = 8). The ages and diagnosed haemophilia in the families of the participant and non-participant carriers are presented in Table 1.

Table 1. Characteristics of the participant and non-participant carriers

	Participants n=126	Non-participants n=144	*P*-value
Age, years (median, range)	48 (21-83)	45 (18-79)	0.02[1]
Haemophilia in family (n, %)			0.10[2]
Severe HA	93 (74 %)	96 (67 %)	
Moderate HA	14 (11 %)	22 (15 %)	
Severe HB	10 (8 %)	15 (10 %)	
Moderate HB	9 (7 %)	11 (8 %)	

HA, haemophilia A; HB, haemophilia B. [1]Mann Whitney U test, [2]Fisher's exact test.

Evaluation of bleeding tendency with the BAT resulted in significantly higher total BS in the carrier group, compared to the control group ($p < 0.01$). The median BS was 2 (range -3 to 12) in the haemophilia A carriers and 3 (range -2 to 12) in the haemophilia B carriers. The median BS in the control group was -1 (range -3 to 8). A BS < 4 was considered to be within the normal range. The BS was < 4 in 82 carriers (65 %) and in all but two women in the control group (98 %). There was no difference in BS between carriers with known haemophilia in the family, compared to carriers with sporadic mutations ($p = 0.92$). The BS was

not correlated to age in the carrier group ($r = -0.07$, $p = 0.44$). There was a negative correlation between age and BS in the control group ($r = -0.40$, $p < 0.001$).

Table 2 shows the proportion of the carriers and controls reporting bleeding symptoms. Each bleeding symptom in the BAT is presented; a score ≥ 1 indicates bleeding symptoms exceeding the normal. The Bonferroni adjustment was applied to account for multiple statistical testing. P-values ≤ 0.004 (p= 0.05/12 tests) were regarded as significant. The carriers reported increased tendency to bleed from tooth extraction and surgery, compared to the controls. The carriers also suffered nosebleeds, cutaneous bleeding, menorrhagia and prolonged bleeding from minor wounds to a greater extent ($p < 0.001$).

Table 2. The frequency of reported bleeding symptoms among participant carriers and controls

	Carriers n (%)	Controls n (%)	*p*-value
Nosebleed	30/126 (24)	4/90 (4.4)	< 0.001
Cutaneous bleeding	22/126 (17)	1/90 (1.1)	< 0.001
Bleeding from minor wounds	40/126 (32)	0/90	< 0.001
Oral cavity bleeding	15/126 (12)	2/90 (2.2)	ns
Gastrointestinal bleeding	6/126 (4.8)	2/90 (2.2)	ns
Tooth extraction	31/104 (30)	0/75	< 0.001
Surgery	25/77 (32)	5/69 (7.2)	< 0.001
Menorrhagia	47/126 (37)	14/90 (16)	< 0.001
PPH	26/108 (24)	7/76 (9.2)	ns
Muscle haematoma	31/126 (25)	18/90 (20)	ns
Haemarthrosis	4/126 (3.2)	2/90 (2.2)	ns
CNS bleeding	1/126 (0.8)	0/90	ns

PPH, post-partum haemorrhage; CNS, central nervous system; ns, non-significant. Fisher's exact test. Bonferroni correction for multiple comparisons: $p \leq 0.004$ was considered significant.

Table 3 presents treatments given to the carriers due to bleeding complications or in order to prevent bleeding. Twenty-four carriers (19 %) had received erythrocyte transfusion on some occasion, compared to four women (4.4 %) in the control group. Furthermore, one woman (1.1 %) in the control group had been given a plasma transfusion and seven (7.8 %) had been treated with tranexamic acid due to bleeding. The controls had not received any prophylactic treatment.

Table 3. Prophylactic treatment and treatment given due to bleeding reported by the carriers

	Prophylactic treatment		Treatment due to bleeding	
Erythrocyte concentrate			24/126	(19 %)
Plasma	1/126	(0.8 %)	4/126	(3.2 %)
FVIII/IX factor concentrate	7/126	(5.6 %)	3/126	(2.4 %)
Desmopressin [1]	15/107	(14 %)	6/107	(5.6%)
Tranexamic acid [2]	26/126	(21 %)	34/126	(27 %)

[1]Only applicable for carriers of haemophilia A. [2]Twelve carriers received tranexamic acid as prophylactic treatment as well as due to bleedings.

4.2 Health-related quality of life in carriers of haemophilia (Paper II)

The respective numbers of carriers and controls who completed the survey were 124 and 90. The entire group of carriers and the subgroup of symptomatic carriers (defined as BS ≥ 4) were compared to the control group. Demographic data and clinical characteristics of the carriers, the subgroup of symptomatic carriers and the controls are presented in Table 4. There was a significant difference in BS between the two carrier groups and the control group ($p < 0.01$). The groups were similar in terms of age, marital status, number of children, education, employment and co-morbidity.

Table 4. Characteristics of the carriers, the subgroup of carriers with increased bleeding tendency and the controls

	Carriers[a] *n* = 124	Carriers, BS ≥ 4[b] *n* = 44	Controls[c] *n* = 90	*p*-value a vs. b	a vs. c
Age, years median (range)[1]	48 (21-83)	48 (21-72)	52 (25-90)	ns	ns
Carriers of HA, n (%)	105 (85)	36 (82)	NA		
Carriers of HB, n (%)	19 (15)	8 (18)	NA		
Family with haemophilia, n (%)	71 (57)	4 (54)	NA		
BS, median (range) [1]	2 (-3 to12)	6.5 (4 - 12)	-1 (-3 to 8)	<0.001	<0.001
BMI > 30, n (%) [2]	22 (18)	9 (20)	8 (8.8)	ns	ns
Current smoker, n (%) [2]	14 (11)	5 (11)	5 (5.6)	ns	ns
Marital status, n (%) [2]				ns	ns
Married/cohabiting	89 (72)	35 (80)	72 (80)		
Single/divorced/widowed	35 (28)	9 (20)	18 (20)		
Children, n (%) [2]				ns	ns
0	11 (8.9)	2 (4.5)	14 (16)		
1-2	87 (70)	32 (73)	53 (59)		
3-5	26 (21)	10 (23)	23 (26)		
Education level, n (%) [2]				ns	ns
Elementary school	33 (27)	13 (30)	16 (18)		
Secondary school	41 (33)	12 (27)	26 (29)		
University	50 (40)	19 (43)	4 8 (53)		
Employment, n (%)[2]				ns	ns
Gainfully employed	97 (78)	38 (86)	79 (88)		
Student	2 (1.6)		1 (1.1)		
Retired	19 (15)	4 (9.1)	10 (11)		
Unemployed	6 (4.8)	2 (4.5)			
Comorbidities, n (%)[2]				ns	ns
Cardiovascular *	11 (8.9)	5 (11)	11(12)		
Diabetes	5 (4.0)	3 (6.8)	2 (2.2)		
Cancer	5 (4.0)	1 (2.3)	5 (5.6)		
Cerebrovascular	1 (0.8)		2 (2.2)		
VTE			1 (1.1)		

HA, Haemophilia A; HB, Haemophilia B; BS, bleeding score; BMI, body mass index; VTE, venous thromboembolism; ns, non-significant. *Cardiovascular includes hypertension. [1]Mann Whitney U test, [2]Fisher's exact test

When comparing the scale scores of the eight SF-36 domains in the entire group of carriers with those in controls, a significant difference was seen in the Mental Health domain (p = 0.048). When the same comparison was made between the group of symptomatic carriers and the control group, there were significant differences in the General Health domain (p = 0.012), the Social Functioning domain (p = 0.021) and the Mental Health domain (p = 0.043). The scale scores in each domain were transformed into Z-scores and compared with the aged-matched Swedish normative female population (n = 4 582). The Z-score represents the difference between the scale score and the normative population

mean, expressed as SD units. A Z-score is negative when the scale score is below the normative population mean and positive when it is above this mean. There was no significant difference between Z-scores in the entire group of carriers and in the control group (Fig 4). There was a significant difference between the Z-scores in the group of symptomatic carriers and the control group in the General Health ($p = 0.008$), Social Functioning ($p = 0.04$) and Mental Health ($p = 0.048$) domains (Fig 5). Each of these differences was, however, less than 0.4 SD from the mean of the normative population.

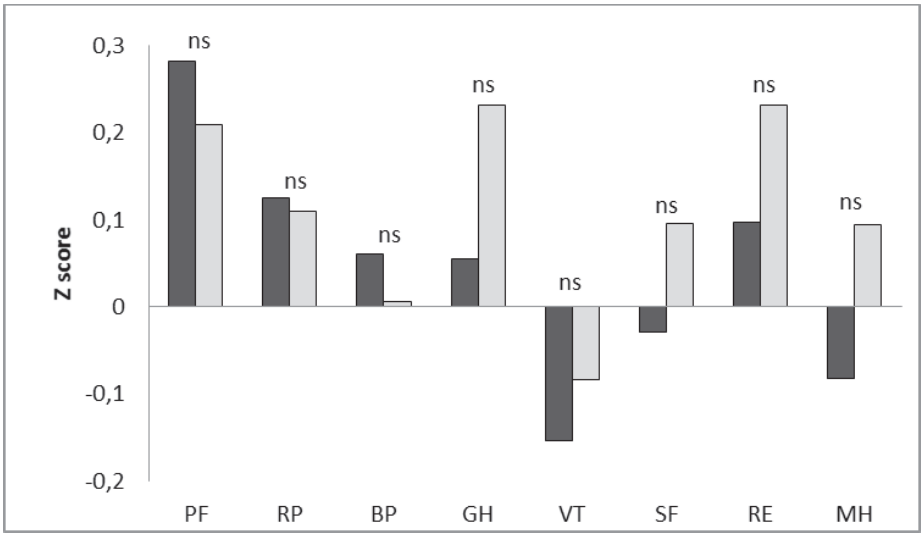

Figure 4. Differences in Z-scores between the entire group of carriers and the control group for each SF-36 domain. Dark bars, carriers; grey bars, controls. The zero line represents Swedish age-matched normative data. PF, Physical Functioning; RP, Role Physical; BP, Bodily Pain; GH, General Health; VT, Vitality; SF, Social Functioning; RE, Role Emotional; MH, Mental Health. Mann Whitney U test.

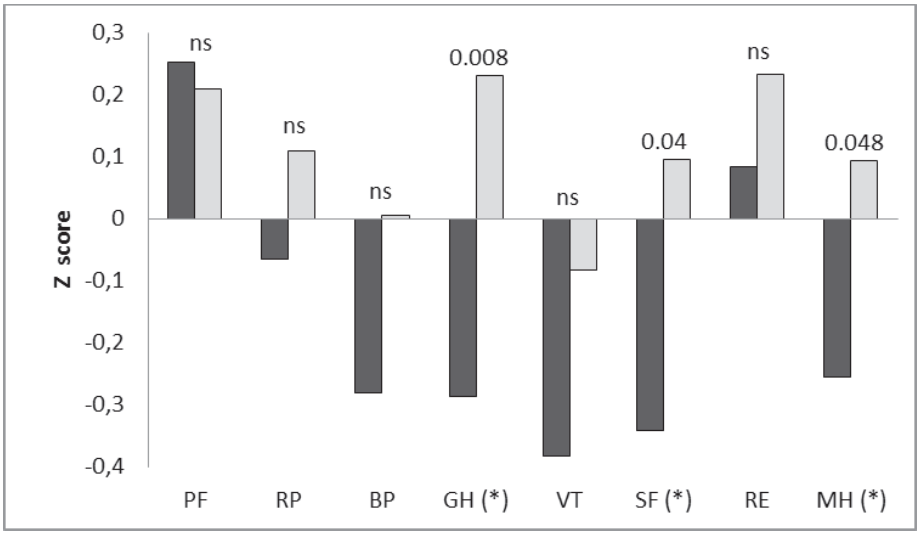

Figure 5. Differences in Z-scores between the group of symptomatic carriers (BS ≥ 4) and the control group for each SF-36 domain. Dark bars, carriers; grey bars, controls. The zero line represents Swedish age-matched normative data. PF, Physical Functioning; RP, Role Physical; BP, Bodily Pain; GH, General Health; VT, Vitality; SF, Social Functioning; RE, Role Emotional; MH, Mental Health. Mann Whitney U test. ($p < 0.05$).*

4.3 Laboratory evaluation of bleeding tendency (Papers I, III and IV)

4.3.1 Factor levels and bleeding tendency

The median FVIII:C level among the haemophilia A carriers was 0.58 kIU/L (intra-quartile range (IQR): 0.42-0.78) and the median FIX:C level among the haemophilia B carriers was 0.50 kIU/L (IQR: 0.33-0.67). A basic laboratory evaluation of haemostasis in the carriers was performed (Table 5). The subgroup of symptomatic carriers (BS ≥ 4) was compared to the subgroup of carriers with a normal bleeding tendency (BS < 4). There was no significant difference between the two groups, including FVIII:C and FIX:C levels.

Table 5. Comparison of laboratory data between carriers with and without increased bleeding tendency

	Carriers, BS < 4 *n* = 82	Carriers, BS ≥ 4 *n* = 44	Reference values[1]	p-value
Haemoglobin (g/L)	133 (127-138)	134 (127-141)	117-153	ns
Platelets (x 10^9/L)	265 (217-306)	262 (223-298)	165-385	ns
PT (INR)	1.0 (1.0-1.1)	1.0 (1.0-1.1)	<1.2	ns
APTT (s)	38 (36-40)	39 (35-42)	30 - 42	ns
Fibrinogen (g/L)	3.1 (2.8-3.6)	3.2 (2.9-3.6)	2.0 - 4.5	ns
FVIII:C (kIU/L)[2]	0.62 (0.44-0.88)	0.52 (0.37-0.71)	0.50 - 2.00	ns
FIX:C (kIU/L)[3]	0.56 (0.45-0.82)	0.36 (0.24-0.62)	0.60 - 1.30	ns
VWF:RCo (kIU/L)	0.98 (0.80-1.28)	1.0 (0.84-1.21)	0.40 - 1.20	ns

[1]Reference values from the Laboratory of Clinical Chemistry, Sahlgrenska University Hospital. [2]Carriers of haemophilia A (n=107), [3]Carriers of haemophilia B (n=19). Results are presented as median and IQR. Mann Whitney U test.

There was a weak correlation between BS and FVIII:C levels in haemophilia A carriers ($r = -0.36$, $p < 0.001$). The BS was not correlated to FIX:C levels in haemophilia B carriers ($r = -0.33$, $p = 0.17$) (Fig 6). Although the factor level of 0.40 kIU/L is the upper limit defining haemophilia, the figure demonstrates an increased BS in a subgroup of carriers with factor levels within the lower normal range (Fig 5). There was a weak correlation between age and FVIII:C levels ($r = 0.24$, $p = 0.015$).The scatterplot (Fig. 7) indicates that the highest FVIII:C levels were found in carriers aged 60 years and up. No correlation was observed between FIX:C and age ($r = 0.39$, $p = 0.10$).

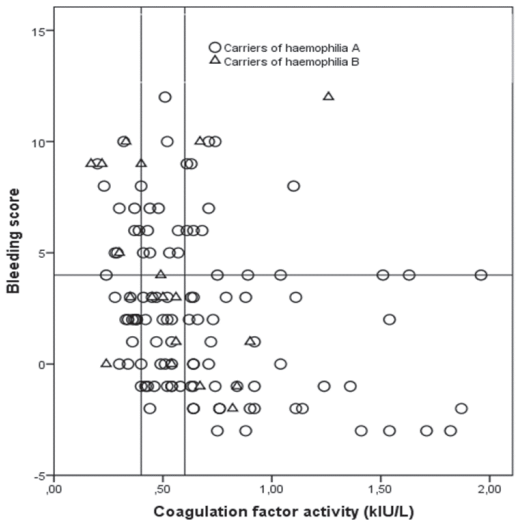

Figure 6. The relationship between coagulation factor activity and BS. Correlation between FVIII:C and BS in carriers of haemophilia A: r = -0.36, p < 0.001. Correlation between FIX:C and BS in haemophilia B carriers: r = -0.33, p = 0.17. Horizontal line indicates BS ≥ 4. Vertical lines indicate coagulation factor activity at 0.40 kIU/L and 0.60 kIU/L.

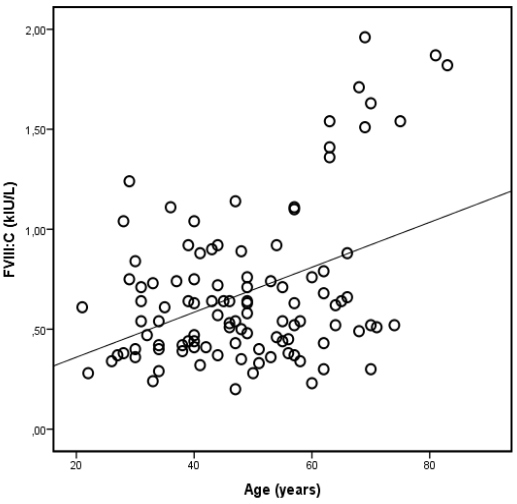

Figure 7. The relationship between FVIII:C levels and age in haemophilia A carriers, r = 0.24, p = 0.015 .

4.3.2 Thrombin generation and bleeding tendency

Since the bleeding tendency among the carriers was not explained by factor levels, the next step was to evaluate in vitro global haemostasis. TG was analysed in platelet-poor plasma (PPP) with the CAT assay. The analysis was performed in haemophilia A carriers (n=106), who were divided into groups according to bleeding tendency. The first group consisted of carriers with BS < 4 (n = 71) and the second group comprised carriers with BS ≥ 4 (n = 35). Fig. 8 shows the results of the ETP and peak thrombin assays, in which 1 pM TF was used as a trigger. There was no significant difference in ETP ($p = 0.52$) or peak thrombin ($p = 0.38$) between the groups of carriers. The carriers were then divided into groups according to FVIII:C level. The first group consisted of carriers with FVIII:C ≥ 0.50 kIU/L (n = 67), while the second group had FVIII:C < 0.50 kIU/L (n = 39). TF 1 pM was used as trigger. The carrier group with low FVIII:C levels had significantly lower ETP ($p < 0.001$) and peak thrombin ($p < 0.01$) values (Fig 9). FVIII:C correlated with both ETP ($r = 0.34$, $p < 0.001$) and peak thrombin ($r = 0.50$, $p < 0.001$). There was no correlation between age and CAT results (data not shown).

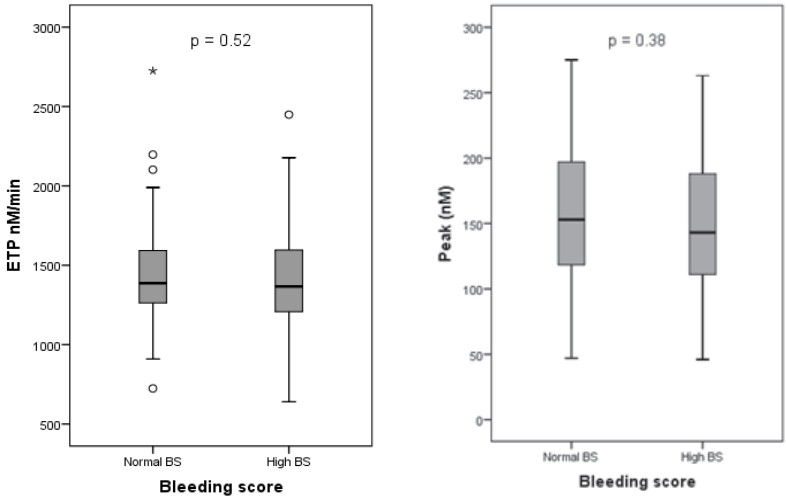

Figure 8. Box plot analyses of the TG (CAT) results (ETP and peak thrombin) in carriers with normal bleeding scores, compared to carriers with increased bleeding scores. Mann Whitney U test.

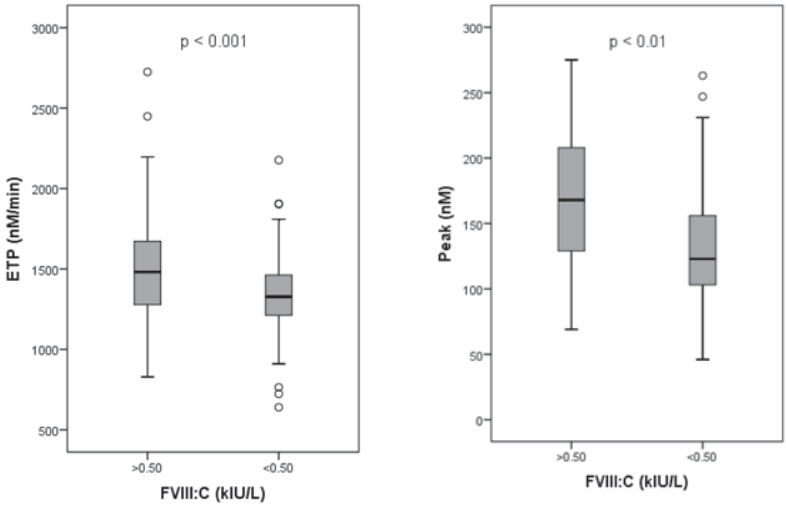

Figure 9. Box plot analyses of the TG (CAT) results (ETP and peak thrombin) in carriers with normal FVIII:C, compared to carriers with low FVIII:C. Mann-Whitney U test.

4.3.3 Genotype and bleeding tendency

The rationale behind dividing the mutations into null and non-null is that a null mutation results in the absence of a gene product or of a gene product functional at the phenotypic level. The F8 gene mutations identified in the haemophilia A carriers are presented in Table 6.

A majority of the null mutations (99 %) were found in carriers belonging to families with severe haemophilia. There were no significant differences in BS between the carriers with null and non-null mutations ($p = 0.37$). There was a significant difference in FVIII:C levels between the carrier groups but, unexpectedly, FVIII:C levels in the null mutation group were significantly higher than those in the non-null group ($p = 0.013$). There was no significant difference in the CAT assay results (peak thrombin and ETP) between the groups.

Table 6. Distribution of F8 mutations in the 106 carriers of haemophilia A

Mutation group (n=106)	**n (%)**
F8 null mutations	
Inversions	51 (48)
Nonsense mutations	8 (7.5)
Small deletions/insertions outside poly-A runs	16 (15)
F8 non-null mutations	
Missense mutations	25 (24)
Small deletions/insertions within poly-A runs	5 (4.7)
Splice site mutations on non-conserved nucleotides	1 (0.9)

5 DISCUSSION

5.1 Methodological considerations

5.1.1 Study sample

Our goal was to identify and investigate the Swedish population of carriers of severe and moderate haemophilia. An initial problem was to locate potential carriers since they seek medical care at hospitals across the country. Carriers with close male relatives diagnosed with haemophilia and carriers with bleeding problems themselves are registered at a HTC to a higher degree. Almost 300 adult potential carriers were identified, of whom approximately half agreed to participate in the studies. It is not unlikely that carriers with a history of bleeding and those with close relatives with haemophilia were more inclined to participate. This selection bias may have resulted in an overestimate of the incidence of bleeding symptoms in the carrier population. Only six of the participating carriers were of non-European origin and the study population was not representative of the Swedish carrier population in that sense. This may have influenced the results since the perception of bleeding may be different due to cultural differences and due to growing up as a haemophilia carrier in a developing country.

5.1.2 Bleeding assessment tool (Papers I-IV)

The evaluation of bleeding tendency is essential to this thesis. The symptoms reported may be influenced by personality, education level and, not least, by family history, since belonging to a family of bleeders may have an impact on how bleeding symptoms are regarded. Moreover, normal subjects report bleeding symptoms as well [82]. The BAT was developed to standardise the diagnostic criteria of type 1 VWD [56] and has subsequently been adapted by investigators to collect data on bleeding in several studies [83-86]. Since we conducted our study, the BAT has been developed further by the ISTH [58]. The current optimized version, the ISTH-BAT, evaluates frequency of bleeding to a larger extent than the severity of each bleeding symptom [58, 87]. Had we used the ISTH-BAT, it might possibly have yielded results with a higher degree of specificity in our cohort of carriers.

5.1.3 Thrombin generation assay (Papers III - IV)

The CAT assay is a research tool and inter-laboratory variability is high. Since our studies were performed, considerable work has been done to produce standardised protocols in an attempt to overcome problems with the method

[88-91]. Protocol standardisation includes the source and concentration of TF and reagents used [77, 92, 93]. Variations in venepuncture procedure and preparation of plasma have also been shown to influence the assay results. The use of corn trypsin inhibitor (CTI), at least at low TF concentrations, is suggested to reduce risk of contact activation through the intrinsic pathway [94-96]. This was not taken into consideration when our analyses were performed.

5.2 Individual papers

5.2.1 Bleeding tendency (Paper I)

The carriers reported more provoked and spontaneous bleedings than the women in the control group. This includes bleeding after surgery, from tooth extraction and menorrhagia, as well as nosebleed, cutaneous bleedings and excessive bleeding from minor wounds. The frequency of PPH was not significantly different between the carriers and the control group, provided that the carriers of haemophilia A and B were not separated. The increased frequency of PPH among haemophilia B carriers can possibly be explained by the increase in FVIII:C, but not FIX:C, that occurs during pregnancy in both carriers and the normal population [97-100]. Earlier studies have demonstrated that carriers with decreased factor levels may have symptoms similar to those in men with mild or, in rare cases moderate, haemophilia [48, 49, 51]. In 2006 Plug et al. published a large study of bleeding tendency in Dutch carriers [52]. Carriers with factor levels within the lower normal range reported increased bleeding tendency, particularly associated with surgery and tooth extractions, compared to carriers with factor levels above 0.60 kIU/L. Our results are in agreement with these findings. A study similar to ours was published in 2014 by Paroskie et al. [83]. The authors reported increased bleeding tendency, in line with our results, when assessing 44 carriers of haemophilia A with a median factor level within the normal range. In conclusion: carriers who phenotypically are defined as having mild haemophilia, but also carriers with factor levels within the lower normal range may have increased bleeding tendency, especially related to haemostatic stress such as surgery and trauma.

5.2.2 Health-related quality of life (Paper II)

The concept of health is defined as physical, psychological and social wellbeing and not merely the absence of disease [101]. Generic instruments are used, irrespective of a specific disorder, and allow comparisons between patient groups and the general population, but they do not yield patterns of symptoms related to specific disorders. The results of our study using the SF-36 questionnaire demonstrated a difference between symptomatic carriers and the

control group. The carriers scored significantly lower in the General Health, Social Functioning and Mental Health domains. However, these differences are within a SD from the mean of the Swedish normative female population. These findings are reassuring, since bleeding generally occurs during specific occasions and not in everyday life. Menorrhagia is an exception, since it is a symptom typically recurring from menarche to menopause in women with increased bleeding tendency. Menorrhagia has been demonstrated to have a negative HRQOL impact in both women with and without increased bleeding tendency [102-104]. The median age of our carriers was 48 years; a large proportion of the women were thus postmenopausal. Data on HRQOL in haemophilia carriers are limited. In a study similar to ours, the authors present a comparison of SF-36 results obtained from 42 haemophilia A carriers and a control group [105]. The carriers scored significantly lower in the Bodily Pain and General Health domains than the control group, but the results were not compared to the normative population. VWD is the most common inherited bleeding disorder. The HRQOL of 317 women with VWD was determined and compared to the normative population in a nationwide Dutch study [106]. A majority of the women had type 1 VWD of moderate severity. The entire population of women with VWD scored significantly lower in the General Health and Vitality domains, compared to the normative Dutch population; however, the Z-scores were lower than 0.3. These data appear to confirm that mild bleeding disorders do not have an impact on HRQOL, compared to the normative population.

5.2.3 Coagulation factor levels (Paper I)

FVIII:C and FIX:C levels are probably not good predictors of bleeding phenotype in carriers of haemophilia [52, 83]. VWD was excluded in our cohort of carriers, but other factors, such as inherited thrombophilia and mild platelet disorders, have not been ruled out as contributors to the phenotype [107, 108]. Furthermore, there is an intra-individual variation in FVIII:C levels in relation to oestrogen levels in women, and reports suggest that FVIII:C levels are highest during the luteal phase of the menstrual cycle [109-111]. The influence on FVIII:C levels due to hormonal therapy depends on the content of oestrogen [112]. However, our study was not designed to take this into account and menstrual cycle status was not recorded. Hormonal fluctuations might have affected FVIII:C levels. The plasma concentration of several coagulation factors, including FVIII:C and FIX:C, increase significantly with age in healthy humans [113, 114]. The carriers in our cohort with FVIII:C levels above 1.50 kIU/L were all more than 60 years of age, which may have influenced the results since the BAT assesses lifelong bleeding tendency.

5.2.4 Thrombin generation assay (Paper III)

Thrombin is a key enzyme in the coagulation system and TG measurement may reflect the in vitro overall coagulation capacity of an individual more accurately than traditional coagulation tests. Studies have demonstrated a relationship between the severity of a bleeding tendency and the ETP, regardless of factor levels. In patients with rare congenital coagulation factor deficiencies, a severe phenotype was associated with an ETP < 20 % of normal [115]. There is an association between phenotype and ETP in haemophilia as well [116, 117]. The TG assay appears to distinguish between bleeding phenotypes in individuals with the same factor levels in both severe and non-severe haemophilia A [118, 119]. We therefore investigated whether bleeding tendency in the haemophilia A carriers could be predicted by the results of the CAT assay. However, the TG potential in symptomatic carriers was not different from that in carriers with normal bleeding tendencies. In comparison to Al Dieri's material, a vast majority of the carriers in our study had milder bleeding tendencies, which presumably contributed to the results. The FVIII:C levels in our study correlated with the ETP and peak thrombin, concurring with studies in the haemophilia setting [77, 120].

5.2.5 Genotype (paper IV)

Type of mutation is a strong predictor of bleeding in men with haemophilia [35, 36, 121]. DNA is, however, primarily tested in potential carriers for the purpose of genetic counselling. The association between type of mutation and bleeding tendency in carriers is not well studied. Miesbach et al. have published data on bleeding tendency, FVIII:C levels and genotype in 43 haemophilia A carriers. The group of carriers with menorrhagia and easy bruising had null-mutations to a higher degree than the asymptomatic carriers [49]. Our results suggest no such association. Indeed, in contrast to men with haemophilia A, carriers have one normal F8 gene producing FVIII. In line with this reasoning, studies have shown conflicting results concerning whether factor levels differ between carriers of severe, moderate and mild haemophilia [44, 122, 123].

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Carriers of moderate and severe haemophilia A and B had increased BS, compared to a control group. Increased bleeding tendency was found not only in a subgroup of carriers with low factor levels but also in a subgroup of carriers with factor levels within the lower normal range. Our findings suggest that it may be beneficial for symptomatic carriers to be followed up at a HTC on a regular basis, in order to prevent excessive bleeding. Our findings also suggest that healthcare professionals need to be aware that women belonging to families with haemophilia might have increased bleeding tendency as well (Paper I).

HRQOL, measured with SF-36, in carriers of haemophilia A and B did not differ from that in the normative Swedish female population. This finding suggests that carriership does not affect HRQOL (Paper II).

TG capacity, measured with CAT, did not differ between symptomatic and asymptomatic carriers of haemophilia A. This finding indicates that CAT may not be a useful tool to assess bleeding tendency or to predict bleeding risk among carriers of haemophilia A (Paper III).

The bleeding tendency in carriers of haemophilia A was not associated with genotype, evaluated by comparison of null and non-null mutations. The genotype in carriers is probably not a good predictor of bleeding tendency since carriers, in contrast to men with haemophilia, have a normal allele producing FVIII to a varying degree (Paper IV).

Further studies to evaluate bleeding tendency and bleeding risk are important in order to provide good care to carriers of haemophilia. Firstly, studies are needed to confirm our results on bleeding tendency, especially in carriers with factor levels within the normal lower range. Secondly, a BAT validated for haemophilia carriers to predict bleeding risk needs to be developed. Thirdly, laboratory methods to evaluate bleeding tendency and predict bleeding risk require improvement. Finally, prospective studies are needed to evaluate haemostasis and carriers' ability to mobilise coagulation factors during and after surgery and delivery. This would be of particular interest in symptomatic carriers with factor levels within the lower normal range.

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APPENDIX

A. Bleeding assessment tool [59]

Symptom	Score					
	-1	0	1	2	3	4
Epistaxis		No or trivial (<5)	>5/year or more than 10'	Consultation only	Packing or cauterization or antifibrinolytic	Blood transfusion or replacement therapy or desmopressin
Cutaneous		No or trivial	>1cm and no trauma	Consultation only		
Bleeding from minor wounds		No or trivial (< 5')	>5/year or > 5'	Consultation only	Surgical haemostasis	Blood transfusion or replacement therapy or desmopressin
Oral cavity		No	Referred at least one	Consultation only	Surgical haemostasis or antifibrinolytic	Blood transfusion or replacement therapy or desmopressin
GI-bleeding		No	Associated with ulcer, portal hypertension, haemorrhoids, angiodysplasia	Spontaneous	Surgical haemostasis, blood transfusion, replacement therapy, desmopressin, antifibrinolytic	
Tooth extraction	No bleeding in at least 2 extractions	None done or no bleeding in 1 extraction	Reported, no consultation	Consultation only	Resuturing or packing	Blood transfusion or replacement therapy or desmopressin
Surgery	No bleeding in at least 2 surgeries	None done or no bleeding in 1 surgery	Reported, no consultation	Consultation only	Surgical haemostasis or antifibrinolytic	Blood transfusion or replacement therapy or desmopressin
Menorrhagia		No	Consultation only	Antifibrinolytics, pill use	Dilation & curettage, iron therapy, ablation	Blood transfusion or replacement therapy or desmopressin or hysterectomy
Postpartum haemorrhage	No bleeding in at least 2 deliveries	None done or no bleeding in 1 delivery	Consultation only	Dilation & curettage, iron therapy, antifibrinolytics	Blood transfusion or replacement therapy or desmopressin	Hysterectomy
Muscle haematomas		Never	Post trauma, no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring surgical intervention or blood transfusion
Haemarthrosis		Never	Post trauma, no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring surgical intervention or blood transfusion
CNS bleeding		Never			Subdural, any intervention	Intracerebral, any intervention

B. SF-36 Measurement model [65]

Scales	Items	
Physical functioning P(PF)	3a. Vigorous activities 3b. Moderate activities 3c. Lift, carry groceries 3d. Climb several flights 3e. Climb one flight 3f. Bend, kneel 3g. Walk mile 3h. Walk several blocks 3i. Walk one block 3j. Bathe, dress	Physical Health
Role-Physical (RP)	4a. Cut down time 4b. Accompished less 4c. Limited in kind 4d. Had difficulty	
Bodily pain (BP)	7. Pain-magnitude 8. Pain-interfere	
General health (GH)	1. EVGFP rating 11a. Sick easier 11b. As healthy 11c. Health to get worse 11d. Health excellent	
Vitality (VT)	9a. Pep/life 9e. Energy 9g. Worn out 9i. Tired	Mental Health
Social Functioning (SF)	6. Social-extent 10. Social-time	
Role-Emotional (RE)	5a. Cut down time 5b. Accomplished less 5c. Not careful	
Mental Health (MH)	9b. Nervous 9c. Down in dumps 9d. Peaceful 9f. Blue/sad 9h. Happy	